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(54) Title: DETECTION OF DNA/RNA BY FLUORESCENCE POLARIZATION

(57) Abstract

A homogeneous method for detecting amplified RNA or DNA target sequences utilizes signal-labelled DNA or RNA probes which show detectably increased fluorescence polarization when hybridized to target sequences. A convenient one-step analytical procedure requiring no nucleic acid extraction or signal separation step is thereby provided.

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DETECTION OF DNA/RNA BY
FLUORESCENCE POLARIZATION

This application is a continuation-in-part of our prior
co-pending application, serial number 7/430,844 filed
5   11/1/89.

Field of the Invention

The present invention relates to the field of
detecting target nucleic acid sequences contained in
complex biological mixtures. More particularly, the
10 invention relates to detection of nucleic acid
sequences originally present in such mixtures n
extremely low concentrations, by first enzymatically
amplifying the specific target sequences, and then
detecting them in a substantially homogeneous assay
15 utilizing fluorescence polarization. An increase in
fluorescence polarization indicates the extent of
hybridization of a probe with the amplified target
sequences.

Background of the Invention

20 In he study of cell populations associated with disease
states it is frequently found that only a subpopulation
of available susceptible cells actually exhibit the
morbid phenotype. In infectious diseases, the
proportions of cells which are passively or actively
25 infected may be very low, and the disease caused by the
infectious agent may go unnoticed clinically even
though an infected individual can transmit the agent to
others. Examples of such infectious agents are clammy,
protozoans, certain bacteria, and many viruses.

30   Amongst viruses, the human immunodeficiency virus
(HIV) is known to have an extremely long latent period
before the onset of the clinical symptoms known as
AIDS. Latency may extend to several years during which
the infected individual is capable of transmitting the
35 virus to others through intimate contact, sharing of

intravenous injection apparatus, or through donation of blood products.

HIV infection is specific for thymus-derived lymphocytes (T cells), and in particular the subset T
5 cells having immune helper function. These T cells possess highly specific HIV receptors on their surfaces to which the virus attaches to gain entry to the cell. Monolingual antibodies, grouped generally in the CD4 cluster, see Leukocyte Typing III, Ed. A.J. McMichael,
10 Oxford University Press, 1987, and specific for the HIV receptor, have been isolated heretofore (see Kung et al., U.S. Patent No. 4,381,295). A signal molecule can be attached to such antibodies which binds selectively to those cells expressing the receptor antigen thereby
15 identifying the helper T cell subpopulation. Quantitation of cell numbers of such lymphocyte subpopulations may conveniently be carried out in a flow cytometer.

In normal individuals approximately 50 percent of
20 peripheral T cells are helper cells. In HIV infected patients, this proportion declines sharply because the virus is cytotoxic to helper T cells. In latent infections or early in the course of clinical disease, the proportion of the helper T cell population actually
25 containing virus, in either lytic or latent phase, may be very low, even to the order of 1 to 1000. This means that in such patients, a 2 ml sample of blood may contain only one or a few copies of the virus or its genome. At his stage of infection, no antibodies to
30 viral proteins can be detected, even with the most sensitive immunological techniques available. There is a great danger that individuals at such early stages of infection may transmit the virus in donated blood without the virus being detected by conventional
35 screening methods.

The most sensitive immunological techniques are capable of detecting antibody by HIV at minimally 21 days post-infection. A variety of immunoassays for detection of HIV have been described including enzyme-

5   linked immunoassays (ELISA), immunodiffusion assays, radioimmunoassays (RIA), and the classical Western blot. Also a number of distinct assay strategies have been developed. One group of assays utilizes HIV viral antigens, particularly viral protein containing

10   epitopes in conserved domains, bound covalently to a solid matrix. The matrix-bound enzyme is contacted with a serum sample, and any anti-antigen antibodies contained therein bind to antigen. In the typical sandwich assay, antiserum raised in a heterologous

15   species against human antibody antigens conjugated to an enzyme (ELISA), fluorescent molecule, or other signal generating substance, is then reacted with the washed matrix-bound antigen-anti-antigen complex. The signal emitted by the signal generating substance is

20   typically a chromophor, fluorescent signal, beta or gamma radiation, or other such measurable emission.
          Alternatively, analysis of serum antibodies maybe obtained by Western blot consisting of gel electrophoresis of viral proteins, electrotransfer of

25   the proteins to blotting paper, followed by reaction with antisera, and color development of the individual protein bands. The Western blot analysis is employed on a confirming test by blood banks in blood screening procedures. For a general review of the various

30   immunological methods available, see Stites et al., <u>Basic & Clinical Immunology</u>, Appleton & Lange, 1987.
          There have been many attempts in the prior art to make detection of serum antibodies to HIV or other low concentration targets more sensitive and selective.

35   The two major approaches have been target amplification

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and signal amplification. In signal amplification, the
object is to couple a very low level signal event to a
large number of subsequent secondary signals which can
be detected and quantified. It is apparent that this
5   coupling must be highly specific so that background
secondary signals do not proportionally increase. One
such signal amplification system takes advantage of the
extremely high affinity of avidin for biotin. A large
number of biotin molecules can be covalently coupled to
10   an antibody specific for viral antigens. When reacted
with fluorochome-coupled avidin a large complex is
formed having unusually brilliant fluorescence.
Another system utilizes a mixture of monoclonal
antibodies conjugated to a signal generating substance,
15   each individual antibody type being specific for a
different structurally distinct epitope. The theory is
that a greater number of signal generating antibody
molecules will bind to antigen if there are no
overlapping specificities.
20   Another approach is to target nucleic acid
sequences of the virus with a homologous nucleic acid
probe coupled to a signal amplification system. Under
renaturing conditions the viral RNA (or denatured DNA)
anneals to the complementary sequence of an
25   oligonucleotide probe. Detection of the hybrid is
afforded by signal generating substances covalently
conjugated to the probe. Applicable to his approach
are the enzyme proteolyzes a zymogen which then acts
upon a substrate to generate a measurable signal. Many
30   of the variations in such techniques are described in
Lelie et al., Detection of HIV Infection Using Second-
Generation HIV Assay, IV International AIDS Symposium,
Stockholm, 1988.
         The second major approach involves target
35   amplification in which the target interacting with the

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signal-generating entity is itself multiplied in
number. Since proteins cannot replicate, target
amplification inherently requires a nucleic acid
sequence, and an enzymatic system which can replicate

5   the target sequence in vitro. One such target
amplification technique is disclosed in U.S. Patent
Nos. 4,683,195 (Mullis et al.) and 4,683,202 (Mullis),
and is called polymerase chain reaction (PCR)
amplification.

10   In PCR, a mixture of nucleic acids containing a
DNA sequence in a small quantity is heated to denature
double stranded DNA. Primers consisting of a
oligonucleotide capable of mediating DNA synthesis from
a single stranded template is added under conditions

15   which favor annealing of the primers to their specific
complementary sequences. A thermostable DNA polymerase
is added, and an extension reaction proceeds at 72°C in
the presence of deoxynucleotide triphosphates,
adenosine triphosphate and cofactors. The reaction is

20   run at high temperatures to avoid non-specific binding
of primer to non-homologous sequences. Under these
stringent conditions fidelity of polymerization to the
desired sequences is very high. .

         After polymerization is complete, the mixture is
25   again heated to denature the double stranded DNAs, and
the extension reaction is repeated. Such repetition of
extension polymerization may occur several times until
the target sequence is amplified in numbers sufficient
to detect by any of the signal-conjugated probe assays

30   described hereinabove.

         In a second target amplification scheme called
TAS, a first primer oligonucleotide or oligonucleotide
containing an RNA transcriptase promoter-binding
sequence is annealed to the target sequence and

35   extended by DNA polymerase or reverse transcriptase.

Following melting, a second primer complementary to the
newly formed oligomer in a region distal to the first
primer binding sequence is added, annealed, and
extended. The resultant duplex DNA oligomer thus has a
5   sequence flanking the target region and containing a
transcriptional promoter. Addition of RNA
transcriptase, in the presence of oligonucleotide
triphosphates, adenosine triphosphate, and cofactors
institutes transcription in vitro yielding up to 1000
10 copies of the target sequence. The TAS methods have
been disclosed in WO 88/01050 (Berg et al.).

In a variation of TAS, RNAse H is added to the
reaction mix. RNAse H specifically catalyzes the step-
wise hydrolysis of RNA bases in an RNA-DNA duplex, so
15 that after a cDNA strand has been synthesized with
reverse transcriptase the RNAse digests the RNA strand
of the duplex to permit synthesis of the second
complementary DNA strand by DNA polymerase. It will be
apparent that since a heating step to melt the DNA-RNA
20 duplex is unnecessary for cyclization of the reaction,
the entire amplification can be performed in a single
incubation. The disadvantages of the TAS and 3SR
methods, compared to PCR is the lesser degree of
stringency because of non-specific primer bonding at
25 the lower temperature.

Finally, target amplification can be carried out
in a ligase-mediated procedure. In this procedure,
complementary primer sets form adjacent hybrids on both
complementary strands of the target. Ligase then joins
30 the primers together at the nick separation, after
hybridization. The ligated double primer can then act
as a template for further ligation of primers in a
subsequent melting and rehybridization step.

After the target amplification, the nucleic acids
35 are ordinarily extracted and the amplified sequences

are detected by the procedures set forth hereinabove.
It should be emphasized that the known procedures of
the prior art are heterogeneous, that is, they require
multiple steps in which the DNA is first hybridized to
5   a signal generating probe, followed by a step in which
the unhybridized probe is separated from hybridized
probe. Ordinarily different sets of reagents are
required for signal generation than for probe
hybridization and separation. A completely homogeneous
10 method for detection of amplified nucleic acids without
a separation step is unknown in the prior art.
Summary of the Invention
      In accordance with the present invention, nucleic
acids amplified by any method are detected in a
15 convenient homogeneous one-step method utilizing
fluorescence polarization. The method of this
invention comprises incubation of denatured amplified
nucleic acids under hybridizing conditions with a
nucleic acid probe of homologous sequence covalently
20 coupled to a fluorophor. The fluorophor-conjugated
probe is added at a concentration sufficient to produce
a measurable increase in fluorescence polarization when
hybridized to the amplified nucleic acid sequences, but
not in an excess quantity so as to appreciable quench
25 the increase in fluorescence polarization attributable
to hybridization.
      It will be apparent that the method of the present
invention is adaptable to a simple automated format for
convenient processing of a large number of samples.
30 This is because there is no nucleic acid extraction
step, and it is unnecessary to separate unhybridized
probe from the mixture. Further, the increase in
fluorescence polarization upon hybridization is
virtually instantaneous, and no repetitious shifts in

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temperature are required in practicing the present
method.

       More particularly, the method of the invention
    comprises: a) Heating or otherwise denaturing a liquid
 5 mixture and for a length of time sufficient to separate
    duplex nucleic acids into single strands, b) adding a
    fluorochrome-conjugated nucleic acid probe having a
    base sequence complementary to a target sequence
    contained in the amplified nucleic mixture, c) lowering
10 the temperature of the mixture for adjusting the pH to
    permit hybridization of probe sequences to amplified
    nucleic acids target sequences; d) incubating the
    mixture for a time sufficient for substantially
    complete hybridization to occur; and e) measuring the
15 degree of fluorescence polarization.

       In a preferred method, target nucleic acid
    sequences are incubated under hybridization conditions
    with a fluorescein-conjugated probe comprising an
    oligonucleotide having substantial complementarity to
20 the target nucleic acid sequences, the fluorescein
    being amino-linked to the oligonucleotide probe through
    an aminochlorotriazinylaminoalkylphosphoryl group,
    incubating for a time sufficient to obtain
    substantially complete hybridization, and measuring the
25 fluorescence polarization. Probes for detecting a
    target nucleic acid sequence are also disclosed which
    comprise a fluorescein-conjugated oligonucleotide
    sequence of substantial complementarity to the target
    nucleic acid sequence, the fluorescein molecule being
30 amino-linked to the oligonucleotide portion through an
    aminochlorotriazinylaminoalkylphosphoryl group. Most
    preferred probes are selected from the group. The
    method of claim 4 wherein the said probes are selected
    from the group consisting of oligonucleotides
35 substantially homologous to target nucleic acids

containing a guanosine or cytosine base in the position
immediately 5' of the base annealing to the
fluorescein-labelled terminal nucleotide of the said
probe.

5   A kit is also contemplated by this invention,
comprising a vessel containing a concentrated buffer
solution of a composition optimizing hybridization of
nucleic acids but without interfering with the
measurement of fluorescence polarization, and a second
10   vessel containing a solution of one or a plurality of
nucleic acid probes conjugated to one or a plurality of
assays. The solutions of the kit of this invention are
readily deliverable step-wise to a multiplicity of
sample containers. The samples can be processed
15   through each of the method steps without transferring
to another container, so that the incubations and
fluorescence polarization determination can be
accomplished in the same vessel and same machine under
automation.

20   Detailed Description of the Preferred Embodiment
        In the technique of fluorescence polarization,
light from a source is utilized to excite fluorescence
emission from a fluorochrome molecule. A fluorophor or
fluorochrome is a molecular entity, usually of
25   molecular weight less than 10,000 daltons which emits
fluorescent light at a characteristic wavelength when
impacted with a radiant energy source. The
fluorochrome is covalently coupled to a nucleic acid
probe hybridizable with a complementary target
30   amplified nucleic acid sequence. A variety of
fluorochromes are known in the art which are adaptable
to the present invention, including fluorescein
derivatives, acidine orange (Van Bertalanffy et al., _J._
Histochem. Cytochem., 4:481 (1956)), benzimidazole
35   derivatives (Hilwig et al., _Exp. Cell Res._, 75:122,

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1972)), and a series of fluorescent nucleic acid
stains, as disclosed in U.S. Patent No. 4,544, 546. In
addition, oligonucleotides bonded to DNA intercalating
agents have been utilized as nucleic acid probes, as
5   disclosed in U.S. Patent No. 4,835,263.

In the preferred embodiment, fluorescein is
covalently attached to an oligonucleotide probe through
an aminochlorotriazinylaminoalkyphosphoryl group having
the generalized structure:

10
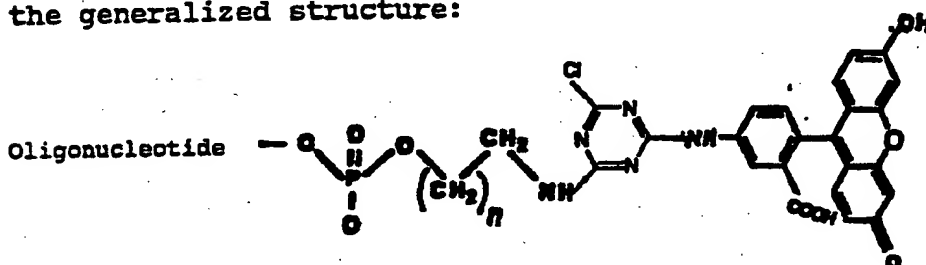
Oligonucleotide

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wherein n is an integer from 2 to about 12 (hereinafter
referred to as DTAF). Most preferred is the
fluorescein conjugted oligonucleotide amino-linked
through an aminochlorotriazinylaminoethylphosphoryl
20   group. It has been determined empirically that
compounds of the preferred generalized structure are
superior in generating signal than other amino-linker
configurations, such as carboxy-fluorescein
succinimidyl ester or the fluorescein isothiocyanate
25   derivative (FITC) having the structure:

Oligonucleotide

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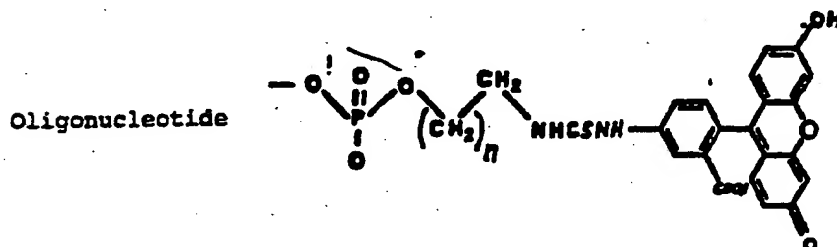
Applicants have further discovered that detection
of hybridization by fluorescence polarization is
substantially enhanced in the preferred DTAF conjugated
35   probe when the target sequence selected contains a

guanosine or a cytosine base in the position
immediately 5' of the base annealing to the
fluorescein-labelled terminal nucleotide of the probe.
The oligonucleotide portion of the probe has a sequence

5   of substantial complementarity to the amplified target
nucleic acids, so that a duplex between probe and
target is formed. It is apparent that perfect
complementarity is not necessary so long as a stable
duplex is formed under the hybridization conditions

10 utilized, and that some base mismatching may be
tolerated. It is further apparent that these probes
may be used in the detection of any substantially
homologous target nucleic acid sequence, and is not
limited to detection of only amplified nucleic acid.

15   The principle of this technique is based upon the
characteristic rotational properties of molecules in
solution. Molecules have a tendency to tumbe about
their various axes of rotation. In general, larger
molecules tumbe more slowly than smaller ones.

20 Compared to the amplified DNA target molecules, the
oligonucleotide fluorochrome-conjugated probes tumbe
very rapidly and along several axes of rotation.
fluorescent light emitted from such spinning molecules
is diffuse and characteristically multiplanar.

25 However, when the probe molecule has annealed to the
target sequence forming a substantially rigid structure
of high molecular weight, it loses much of its spin.
As a result fluorescence emission becomes relatively
polarized. The emission fluorescence polarization of

30 solution in which intercalating fluorochrome binds iwth
DNA was described by Arschine et al., The EMBO J., 3,
795 (1984). This polarization effect can be measured
conveniently in a fluorometer and a polarizatin value
is calculated accordingly to the following equation:

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$$P = \frac{I_{PA} - I_{PE}}{I_{PA} + I_{PE}}$$

wherein P is polarization units, I_{PA} is the parallel

5   intensity and I_{PE} is the perpendicular intensity.

The oligonucleotide portions of the probe may have
a variable number of bases, preferably about 10-40
which are substantially homologous with the target
sequence. For generic identifications, it is important

10   to select highly conserved regions of the genome so as
to ensure substantial homology between different
species, or genetic variants of the same infectious
organism or other nucleic acid target. Further
increased sensitivity of the present method may be

15   attained by sue of more than one probe specific for
different amplified sequences. In the case of HIV,
selection of conserved sequences is an important
consideration because the envelope proteins are
genetically labile and mutant variations occur

20   extremely rapidly. This has been found to result from
replication errors. Reverse transcriptase is a highly
imprecise replicator. On the other hand, since this
property of the enzyme is particularly functional, the
sequence encoding the enzyme itself is highly

25   conserved. Accordingly, the sequences encoding the
reverse transcriptase make excellent oligonucleotide
probes which can be expected to hybridize to virtually
any HIV variant. Conversely, selection of probes of
short hybridizable sequences, preferable of 6 to 12

30   bases, from highly mutogeneic genomic regions may be
utilized diagnostically to assess the degree of drift
in homology over a period of time, or between different
patient hosts.

As indicated hereinabove, there are now a number

35   of different methods for amplifying nucleic acids.

Once amplification has occurred, whether by PCR. TAS,
3SR, or LAS, the method of the present invention is
equally applicable t detection of the nucleic acid so
amplified, without regard to the amplification method
5   used. It will be apparent to those skilled in the art
that the inventive method of detection will be
applicable as well to any future improvement or new
technology in the nucleic acid amplification art.

The buffer utilized during hybridization may be
10   selected from the group of buffers having buffering
agents with a pH in the range of 6.5 to 8, having an
ionic strength of 100 500 mM, and having 10 to 100 mM
of a chelating substance. A typical buffer (20x
concentration) is described in Maniatis et al.,
15   Molecular Cloning, 1982 and contains 200 mM sodium
monobasic phosphate, 3 M sodium chloride, 20 mM
ethylene diamnetetracetic acid, at pH 7.4 It was found
empirically that this buffer provides good conditions
for hybridization without appreciable quenching or
20   other deleterous effect on the fluorescence
polarization detection step.

The kit of the present invention provides a first
vessel in which amplification of specific nucleic acid
is done in a small volume. The amplified nucleic acid
25   is then denatured by boiling or by the addition of
sodium hydroxide. A second vessel of the kit contains
the probe in a reconstitutable, lyopholozed, or
concentrated liquid form which also contains
hybridization buffer. The second vessel of the kit is
30   made of a substance which does not readily absorb
oligonucleotide molecules to its surfaces. Examples of
such materials are polyethylene, polypropylene, teflon,
silanized or siliconized glass. Finally, the opening
of the vessels may have flange or valve means whereby
35   to facilitate drawing of liquids contained therein into

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lines conveying the liquids to an automated machine for
processing of a large number of amplified nucleic acid
samples. Other advantages of the present invention
will be apparent from the Examples which follow.

5   Examples

In initial experiments, the target sequence
selected for study was a section of the HIVPV22
sequence partially encoding reverse transcriptase of
HIV. A section of this sequence (bases 1195-2690) was
10 cloned into plasmid p24L utilizing conventional
techniques. The PCR reactions on both the plasmid and
DNA from infected cells, termed HIV-DASH amplification
region. The oligonucleotide primers at the 3' and 5'
termini are shown on the figure. Upon denaturation of
15 HIV-DASH and annealing of the primers to their target
sequences, DNA polymerase, preferably a highly purified
form of recombinant enzyme from which the endonuclease
encoding sequences have been deleted, is added to
obtain chain extension. This procedure was repeated
20 several times. This method of PCR amplification does
not depart appreciably from the method disclosed in
U.S. Patent Nos. 4,683,145 and 4,683,202, and
variations reported in the literature.

Upon completion of amplification, he nucleic acids
25 are once again denatured. Denaturation can be effected
thermally by boiling or, preferably, by addition of
NaOH at 55°C resulting in a pH of approximately 13.
The fluorochrome-conjugated oligonucleotide probes are
added. If denaturation was effected by boiling,
30 renaturation will proceed by simply lowering the
temperature to 37° - 48°C. If denaturation was
effected at high pH, then a sufficient amount of Tris
pH 5.5 buffer is added to reduce the pH to 8, so that
hybridization of probe proceeds.

15

Empirically, it was discovered that signal
detection is enhanced when complementary probe pairs
are employed, and also when more than one target
sequence is used. Also, it was found that surprisingly

5   a much better detection signal was generated in
relatively large volumes of low target DNA
concentration. Accordingly, the experiments of these
examples were conducted in 1.5 - 2.0 ml volumes instead
of 50 microliter microassays.

10   In the experiment of Example 1, reagents comprised
a probe designated DASH3-AMI-FITC which has the
oligonucleotide sequence shown in Figure 2, covalently
attached through an amide linkage to fluorescein
isothiocyanate designated DASH3, and a second

15   unconjugated oligonucleotide sequence complementary to
DASH3-AMI-FITC, designated DASHIC, and a third
unconjugated oligonucleotide sequence, designated
DASH#, as a noncomplementary control. These reagents
were added together as follows: with 1.9 ml 5x SSPE

20   buffer (750 mM NaCl, 10 mM sodium phosphate monobasic,
1 mM EDTA), 100 microliters 120 mM DASH3-AMI-FITC in 5x
SSPE buffer, 5, 20, 50, 100 microliters respectively,
in separate tubes; 100 mM DASH3c in 5x SSPE buffer, or
alternatively, 5, 20, 50, 100 microliters 100 mM DASH3

25   in 5x SSPE, respectively in separate tubes. Background
fluorescence polarization values were obtained for each
tube immediately after addition of the DASH3-AMI-FITC
and before addition of the other probes. After the
hybridization reactions were begun, fluorescence

30   polarization was monitored over a 35 minute period.
The results are shown in Figure 3 of the drawings.
It is readily apparent that hybridization of the
fluorochrome-conjugated probe with its complementary
probe sequence produces substantial polarization of

35   fluorescence, and that the amount of polarization is

substantially quantitative. This is essentially a
control experiment demonstrating that hybridization to
the relatively short complementary sequence brings
about a significant degree of molecular spin

5   inhibition.

Example 2

In another experiment, of substantially identical
format, the capture target sequence for labelled probe
is a PCR amplified HIV target derived from

10   amplification of the p24L plasmid known to contain the
DASH sequence. The results are shown in Figure 4 of
the drawings. Hybridization was monitored over 55
minutes and carried not in a total reaction volume of
1.6 ml. The glossary of graph points on the right side

15   of the figure expresses the DNA content of the reaction
mix as the number of tipomoles of pre-PCR DNA present
prior to the amplification step and, correspond to 0,
10^2, 10^3, 10^4, and 10^5 pre-amplification molecules of
p24L plasmid DNA. Denaturation was carried out by

20   incubating 20 microliters PCR reaction mix with 25
microliters water and 5 microliters NaOH at 55°C for 15
minutes. Tubes containing 1.5 ml 5x SSPE buffer, 7
microliters of 3.2 M Tris-Hcl and 100 microliters
DASH3-AMI-FITC (10 mM in 5x SSPE buffer) were prepared

25   and background fluorescence polarization measured.
Fifty microliter aliquots of the PCR-DNAs were added
and fluorescence polarization measured at the times
indicated.

The results graphed in Figure 4 of the drawings

30   clearly show an increase in fluorescence polarization
which is generally dose-dependent with increasing
amounts of added PCR-DNA. This is an especially
significant finding because of the large excess of DNA
present which is noncomplementary to the DASH sequence

35   in this homogeneous assay.

Example 3

In a still further experiment the RNA fraction of H-9 cells infected with HIV virus was extracted by conventional methods. A portion containing 0.1
5   attomoles of HIV RNA was then amplified utilizing the 3SR amplification techniques described hereinabove. Test tubes for assay of probe-RNA hybrids were prepared by adding 1.5 ml 5x SSPE buffer to 100 microliters of a 10 mM DASH3-AMI-FITC solution. 3SR RNA was alkalie
10 denatured. Sixty microliters of the denatured 3SR amplified RNA was added to the test tubes, and fluorescence polarization was monitored over a 45 minute time period. The results, shown in Figure 5, indicate that 3SR amplified RNA derived from the RNA
15 fraction of HIV infected cells gives a strong, unequivocal positive signal compared to an unamplified control. This example illustrates that the detection method of the present invention is a versatile assay of nucleic acids containing target sequences amplified by
20 any method.

Example 4

In the experiment of this example, the resolving power of the present method was evaluated. The supernatant from a growing culture of H-9 cells
25 infected with HIV was obtained and the DNA putatively contained therein was subjected to 60 cycles of PRC. The amounts of supernatant amplified corresponded to a volume of cultured cells containing 10^2, 10^3, 10^4 cells. Similarly, identical control supernatants were
30 prepared from growing cultures of non-infected H-9 cells. Test tubes were prepared containing 1.5 ml 5x SSPE buffer, 5 microliters 1N NaOH, 7 microliters 3.2 M Tris pH 5.5, and 100 microliters of 10 mM DASH3-AMI-FITC. Target DNA was denatured by adding 5 microliters
35 1N NaOH and, 25 microliters water to 20 microliters PCR

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reaction mix followed by incubation at 55°C for 15
minutes. Fifty microliters of the denatured PCR
reaction solution was added to the test tubes
containing the DASH3-AMI-FITC probe and hybridization
5   proceeded with monitoring by fluorescence polarization.
Figure 6, shows that affirmative HIV sequence detection
can be obtained of the PCR amplification of the
supernatant containing as few as 200 cells. This means
that the method of the present invention has sufficient
10   sensitivity and resolving power to be useful as a
routine serum screening assay for individuals with very
early pre-clinical HIV infection.

In practicing the present invention, it must be
emphasized that the probe technology and hybridization
15   conditions are critical. The target sequences must
have high binding affinity to probe and be highly
specific for host sequences. Purity is also an
important factor. Also, the fluorochrome-conjugated
probe sequence length must be short enough that the
20   rotational and relaxational properties show contrast in
fluoroscence polarization values when hybridized.
Specific probe sequence may influence the rate at which
annealing of probe to target sequence occurs, compared
to the rate at which reannealing of native
25   complementary target regions occurs. While is does
appear that the amino-linker probe coupling strategy
utilized in these examples is particularly efficacious,
o⁄ r linkage modes may theoretically be substituted by
and error.

30   ᴎple 5

In these experiments the probe and target reagents
were as follows: a probe designated DASH3-15-AMI-FITC
which has the oligonucleotide sequence shown in Figure
7 covalently attached through an amino-linker arm to
35   fluorescein isothiocyanate, a second probe designated

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DASH3-15-AMI-DTAF whch has an identical sequence as the
above probe but covalently attached through an amino-
linker arm to dichlorotrianzinylaminofluorescein, and
an unconjugated oligonucleotide target sequence
5   designated T23-DASHIC which has the sequence shown in
Figure 7 and which is complementary to both of the
above probes. In separate tubes each probe was added
to the target sequence and fluorescence polarization
was monitor as in Example 2. The results tabulated in
10   Table 1 clearly shown that upon hybridization to a
complementary target sequence, a probe labelled with
DTAF gives higher changes in fluorescence polarization
(delta mP) than a probe labelled with FITC, upon
hybridization to a complementary target sequence.

15   Table 1

                FLUORESCENCE POLARIZATION (mP)

                    Pre-   Post-
        PROBE   hybridization   hybridization   Delta
    DASH-15-AMI-FITC   93.0   169.8   76.8
20   DASH-15-AMI-DTAF   111.8   240.1   128.3

        In further experiments the probe and target were
as follows: a probe designated DASH3-15-AMI-DTAF which
has the oligonucleotide sequence shown in Figure 8
covalently attached through an amino-linker arm to
25   dichlorotrianzinylaminofluorescein, and five
unconjugated oligonucleotide target sequence designated
T23-DASHIC-G, T23-DASHIC-A, T23-DASHIC-T, and T23-
DASHIC-C which have the sequences shown in Figure 8 and
are complementary to the above probe. In separate
30   tubes the probe was added to each of target sequences
and fluorescence polarization was monitored as in
Example 2. The results, graphed in Figure 9 clearly,
shows that the nucleotide of target sequence adjacent
to the probes' linker arm after the hybridization
35   affects the degree of fluorescence polarization.

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Greater changes (delta mP) in fluorescence polarization
are obtained if the said nucleotide is a deoxycytidine
(C) or a deoxyguanidine (G).

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WHAT IS CLAIMED IS:

1.   The method of detecting amplified nucleic acids
     comprising:

          a) incubating denatured amplified nucleic
5   acids with a fluorochrome-coupled nucleic
          acid probe of complementary base sequence,
          whereby to hybridize said amplified nucleic
          acids to said probe, and

          b) measuring the fluorescence polarization of
10   said hybridized nucleic acids and probe.

2.   The method of detecting amplified nucleic acids
     comprising:

          a) denaturing a liquid mixture containing
          amplified nucleic acids, by heating to a
5   temperature and for a length of time
          sufficient to separate duplex nucleic acids
          into single strands;

          b) adding a fluorochrome-conjugated nucleic
          acid probe having a base sequence
10   complementary to a target sequence contained
          in the amplified nucleic mixture;

          c) lowering the temperature;

          d) incubating the mixture for a time
          sufficient for substantially complete
15   hybridization to occur; and

          e) measuring the degree of fluorescence
          polarization.

3.   The method of detecting amplified nucleic acids
     comprising:

          a) denaturing a liquid mixture containing
          amplified nucleic acids by raising the pH;

5   b) adding a fluorochrome-conjugated nucleic
          acid probe having a base sequence
          complementary to a target sequence contained
          in the amplified nucleic mixture;

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    c) adjusting the pH to permit hybridization

10   of probe sequences to amplified nucleic acids

    target sequences;

    d) incubating the mixture for a time

    sufficient for substantially complete

    hybridization to occur; and

15   e) measuring the degree of fluorescence

    polarization.

4.   The method of claim 1, 2, or 3, wherein said probe
oligonucleotide contains 8 to 40 bases.

5.   The method of detecting amplified target nucleic
acids comprising:

    incubating amplified nucleic acids with a
fluorescein-conjugated oligonucleotide probe

5   having a sequence of substantial complementarity
to the said target nucleic acids, said fluorescein
being amino-linked to said probe through an
aminochlorotriazinylaminoalkylphosphoryl group
incubating for a time sufficient for substantially

10   complete hybridization to occur;

    and measuring the degree of fluorescence
polarization.

6.   The method of claim 4 wherein the said probes are
selected from the group consisting of
oligonucleotides substantially homologous to
target nucleic acids contianing a guanosine or

5   cytosine base in the position immediately 5' of
the base annealing to the fluorescein-labelled
terminal nucleotide of the said probe.

7.   A probe for detecting a target nucleic acid
sequence comprising

    a fluorescein-conjugated oligonucleotide
having a sequence of substantial complementarity

5   to the said target nucleic acid sequence, said
fluorescein being amino-linked to said probe

through an
aminochlorotriazinylaminoalkylphosphoryl group.

8. The probe of claim 6 wherein said probes are
selected from the group consisting of
oligonucleotides substantially homologous to
target nucleic acids contianing a guanosine or
5   cytosine base in the position immediately 5' of
the base annealing to the fluorescein-labelled
terminal nucleotide of the said probe.

9. The method of claim 1, 2, or 3, wherein said probe
oligonucleotide sequence is derived from the HIV
genome sequence encoding reverse transcriptase.

10. The method of claim 1, 2, or 3, wherein said
amplified nucleic acids were amplified by the PCR
technique.

11. The method of claim 1, 2, or 3, wherein said
amplified nucleic acids were amplified by the TAS
technique.

12. The method of claim 1, 2, or 3, wherein said
amplified nucleic acids were amplified by the 3SR
technique.

13. The method of claim 1, 2, or 3, wherein said
amplified nucleic acids were amplified by the LAS
technique.

14. A kit for detecting amplified nucleic acids
comprising:
a) a first vessel containing a hybridization
buffer, and
5   b) a second vessel containing a fluorochrome-
coupled oligonucleotide probe.

FIG. 1

```
2201   CAGCCCCACC AGAAGAGAGC TTCAGGTCTG GGGTAGAGAC AACAACTCCC

2251   CCTCAGAAGC AGGAGCCGAT AGACAAGGAA CTGTATCCTT TAACTTCCCT

2301   CAGATCACTC TTTGGCAACG ACCCCTCGTC ACAATAAAGA TAGGGGGGCA

2351   ACTAAAGGAA GCTCTATTAG ATACAGGAGC AGATGATACA GTATTAGAAG

2401   AAATGAGTTT GCCAGGAAGA TGGAAACCAA AAATGATAGG GGGAATTGGA

2451   GGTTTTATCA AAGTAAGACA GTATGATCAA ATACTCATAG AAATCTGTGG

2501   ACATAAAGCT ATAGGTACAG TATTAGTAGG ACCTACACCT GTCAACATAA

2551   TTGGAAGAAA TCTGTTGACT CAGATTGGTT GCACTTTAAA TTTTCCCATT

2601   AGCCCTATTG AGACTGTACC AGTAAAATTA AAGCCAGGAA TGGATGGCCC

2651   AAAAGTTAAA CAATGGCCAT TGACAGAAGA AAAAATAAAA GCATTAGTAG

2701   AAATTTGTAC AGAAATGGAA AAGGAAGGGA AAATTTCAAA AATTGGGCCT

2751   GAAAATCCAT ACAATACTCC AGTATTTGCC ATAAAGAAAA AAGACAGTAC
```

FIG. 2

HIV-DASH SEQUENCE

```
        |_____5' PRIMER_____|
        |                         |
        |10        20        30        40        50
TTAGATACAG GAGCAGATGA TACAGTATTA GAAGAAATCA GTTTGCCAGG
AATCTATGTC CTCGTCTACT ATGTCATAAT CTTCTTTAGT CAAACGGTCC


                            DASH3
              |_____(DASH3-AM1-FITC) _____|
              |                                |
        60        70        80        90        100
AAGATGGAAA CCAAAAATGA TAGGGGGAAT TGGAGGTTTT ATCAAAGTAA
TTCTACCTTT GGTTTTTACT ATCCCCCTTA ACCTCCAAAA TAGTTTCATT


              |_____      DASH1C        _____|
              |                                  |
        110       120       130       140       150
GACAGTATGA TCAGATAGTC ATAGAAATCT GTGGACATAA ACCTATAGGT
CTGTCATACT AGTCTATCAG TATCTTTAGA CACCTGTATT TGGATATCCA


        160       170       180       190       200
ACAGTATTAG TAGGACCTAC ACCTGTCAAC ATAATTGGAA GAAATCTGTT
TGTCATAATC ATCCTGGATG TGGACAGTTG TATTAACCTT CTTTAGACAA


        210       220       230       240       250
GACTCAGATT GGTTGCACTTT AAATTTTCC CATTAGCCCT ATTGAGACTGT
CTGAGTCTAA CCAACCTGAAA TTTAAAAGG GTAATCGGGA TAACTCTGACA


        260       270
ACCAGTAAAA TTAAAGCCAGG AATGGAT
TGGTCATTTT AATTTCGGTCC TTACCTA

____3' PRIMER_____|
                     |
```
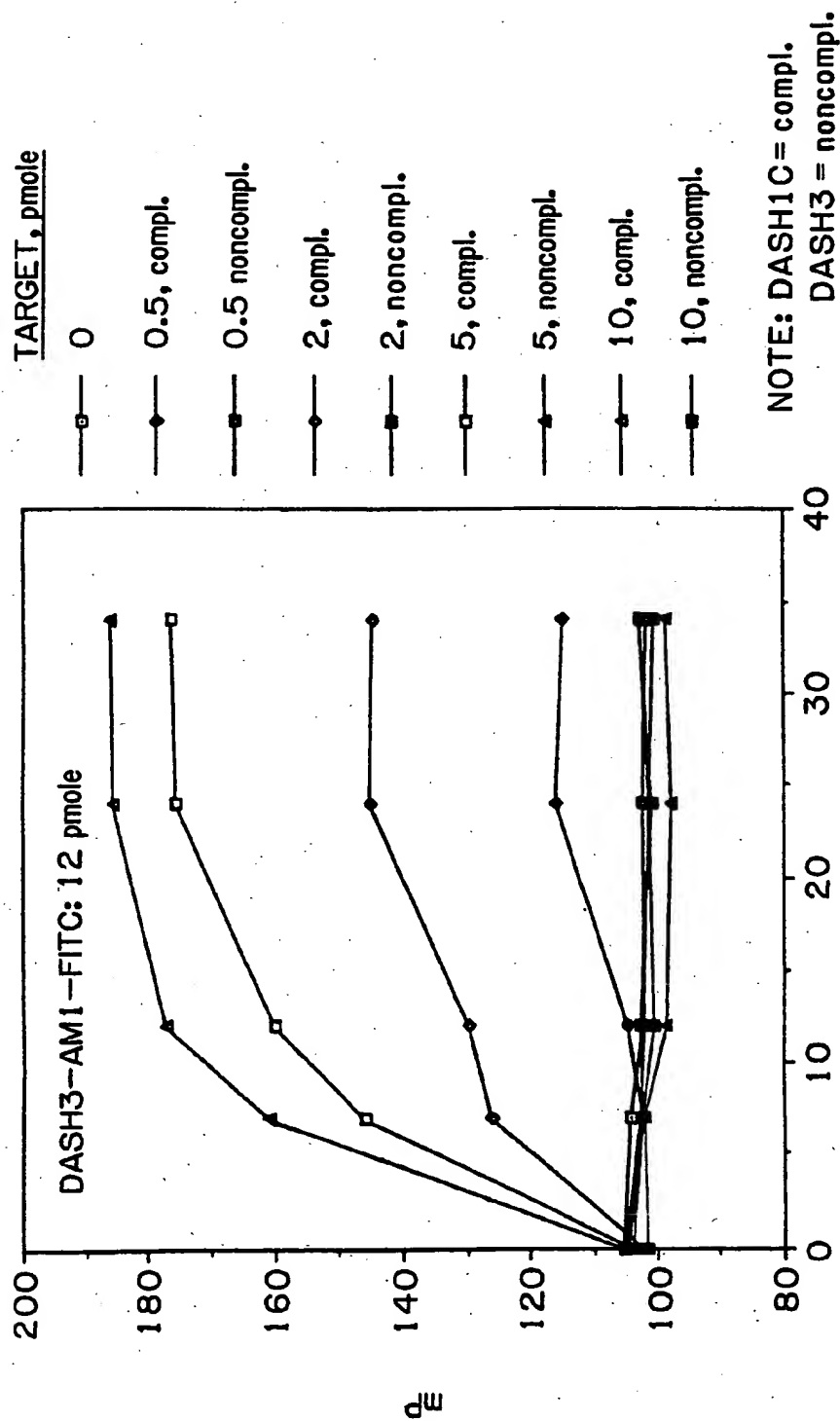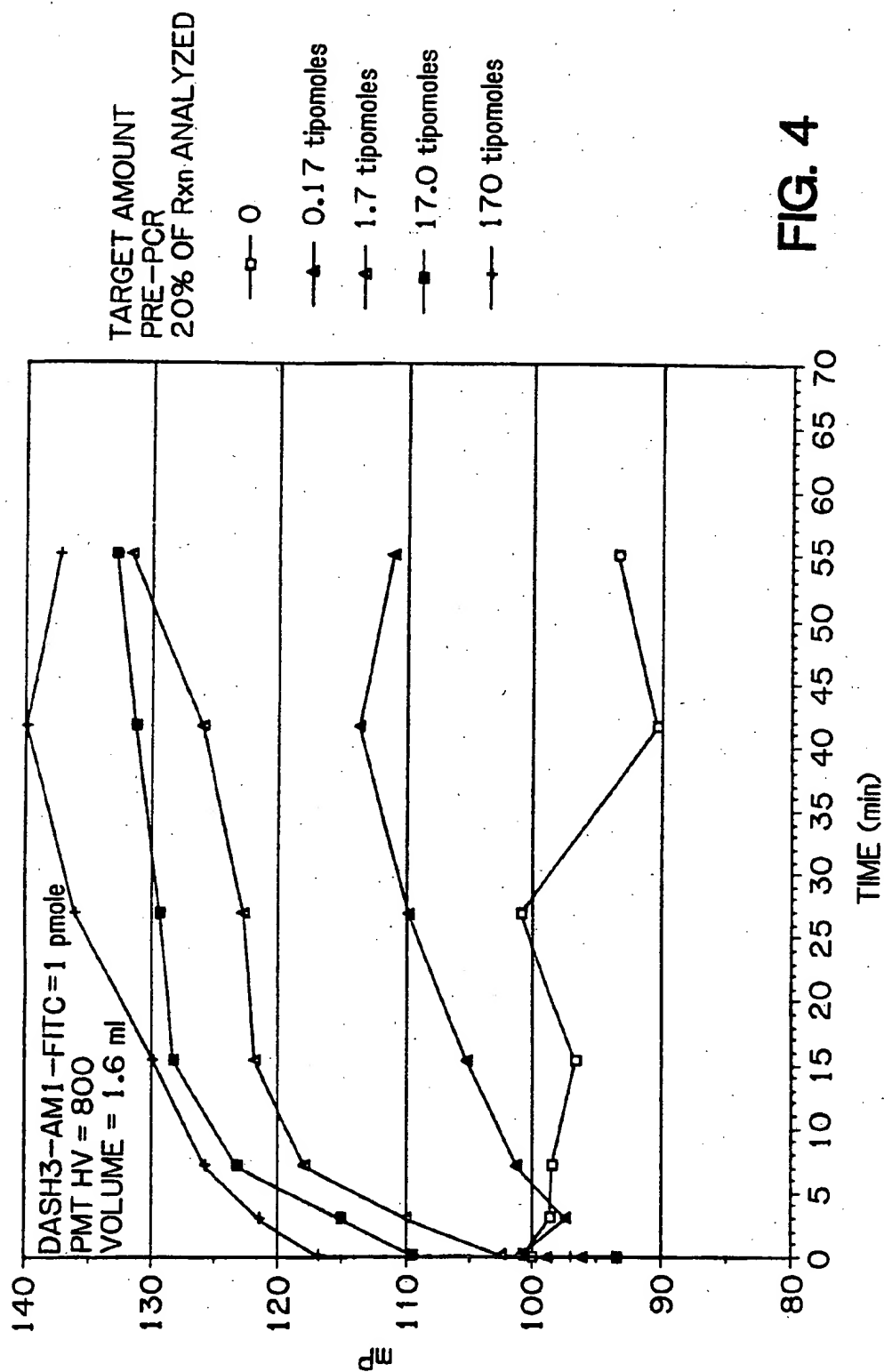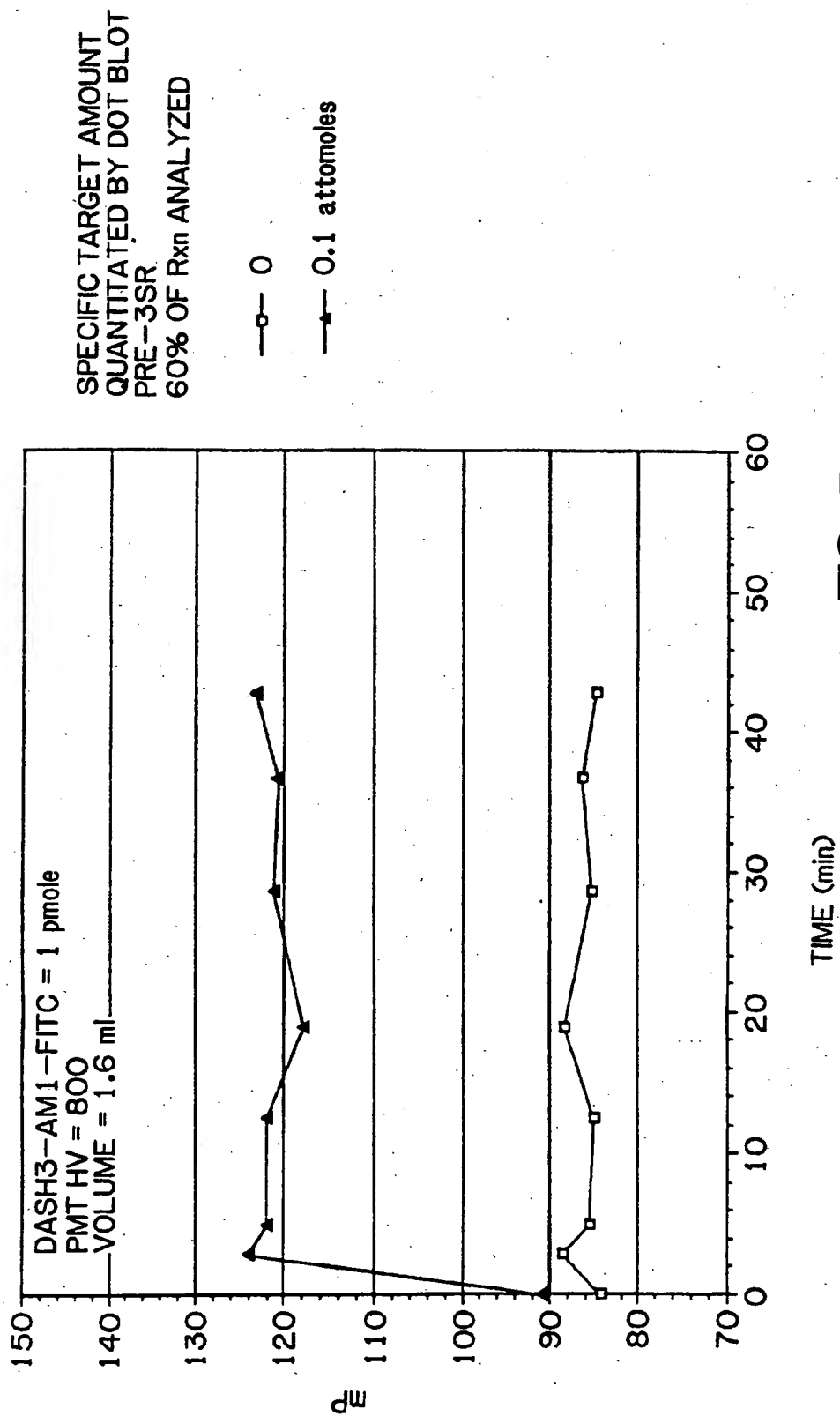
FIG. 3

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FIG. 4

SPECIFIC TARGET AMOUNT
QUANTITATED BY DOT BLOT
PRE–3SR
60% OF Rxn ANALYZED

□ 0

▲ 0.1 attomoles

DASH3–AM1–FITC = 1 pmole
PMT HV = 800
VOLUME = 1.6 ml

mP

TIME (min)

FIG. 5

FIG. 6

FITC 5'
G A T A G G G G G A A T T G G 3'
DASH3-15-AM1-FITC

DTAF 5'
G A T A G G G G G A A T T G G 3'
DASH3-15-AM1-DTAF

3' G G T T T T T A C T A T C C C C C T T A A C C 5'
T23-DASH1C

FIG. 7

DTAF 5'
G A T A G G G G G A A T T G G 3'
DASH3-15-AM1-DTAF

3' G G T T T T T G C T A T C C C C C T T A A C C 5'
T23-DASH1C-G

3' G G T T T T T A C T A T C C C C C T T A A C C 5'
T23-DASH1C-A

3' G G T T T T T T C T A T C C C C C T T A A C C 5'
T23-DASH1C-T

3' G G T T T T T C C T A T C C C C C T T A A C C 5'
T23-DASH1C-C

FIG. 8

FIG. 9

*Note: These are post hybridization values

INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(5)   :C12Q 1/68; C07H 15/12; C12N 15/00
US CL   :435/6; 536/27; 935/77,78

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/6; 536/27; 935/77,78

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
y	American Biotechnology Laboratory, Volume 18, No. 13, issued 1990, Kwoh et al, "Target Amplification Systems in Nucleic Acid-Based Diagnostic Approaches", pages 14-25, see entire document.	1-14
y	Ann. Biol. Clin., Vol. 48, issued 1990, Gingeras et al, "Unique Features of the Self-Sustained Sequence Replication (3SR) Reaction in the in Vitro Amplification of Nucleic Acids", pages 498-501, see entire document.	1-14
y	US, A, 4,820,630 (Taub).11 April 1989, see especially column 5, lines 16-20, and column 7, lines 16-21.	1-14
y	US, A, 4,683,202, (Mullis) 28 July 1987, see entire document.	1-14

☐   Further documents are listed in the continuation of Box C.   ☐   See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 JULY 1992	1 7 JUL 1992

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